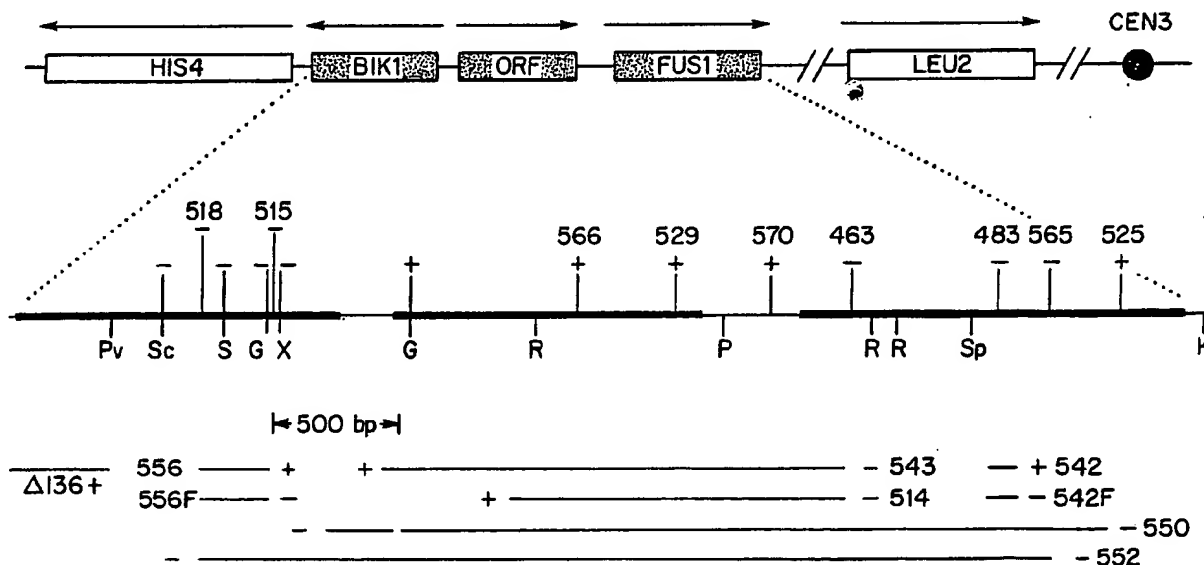




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(54) Title: **PHEROMONE-INDUCIBLE YEAST PROMOTER**

(57) Abstract

A yeast promoter inducible by the appropriate pheromone and a method of expressing a gene of interest in substantial quantities by placing it under the control of the inducible promoter. DNA encoding a protein of interest is fused or linked to a pheromone-inducible yeast promoter, such as the *FUS1* or the *FUS2* promoter, and the fusion is inserted onto a high copy vector; the resulting product is introduced into wild type yeast cells. Stimulation of these yeast cells by the appropriate pheromone results in induction of transcription of the yeast promoter and expression of the protein of interest in substantial quantities.

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PHEROMONE - INDUCIBLE YEAST PROMOTERDescriptionBackground

05 It is possible, using recombinant DNA technology, to
clone and express in bacteria and yeast a variety of
genes which are not present in such organisms as they
occur in nature. For example, procedures for cloning DNA
segments in E. coli, by inserting the DNA into a plasmid
or bacteriophage genome, are well established and
10 frequently used to isolate prokaryotic and eukaryotic
genes.

The yeast Saccharomyces cerevisiae (S. cerevisiae)
is also used as a host for heterologous gene expression
and protein secretion. Availability of techniques which
15 make it possible to introduce exogenous DNA into yeast
genomic DNA has made it possible to develop yeast strains
which produce and secrete foreign proteins such as alpha
interferon, epidermal growth factor, calf prochymosin and
beta-endorphin. Heterologous eukaryotic genes can be
20 expressed in S. cerevisiae if they are placed under the

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control of a yeast gene promoter region. Heterologous protein yields will be determined, at least in part, by the promoter chosen. As an example, the promoter from the yeast gene for phosphoglycerate kinase (PGK) has been described as directing expression of heterologous genes with efficiency at least 500-fold greater than that evident when the TRP1 promoter is used. Mellor, J. et al., Gene, 33:215-226 (1985). Hitzeman and co-workers describe work to increase expression of the gene encoding bovine growth hormone in yeast. The 5'-promoter region, translation signal and signal peptide sequences were replaced with yeast genomic DNA from similar regions. Hitzeman, R. A. et al., Nature, 295:717-722 (1981).

The type of vector chosen (e.g., a high-copy-number 2 micron plasmid-based shuttle vector, rather than an unstable high-copy-number ARS-based plasmid or a low-copy-number stable ARS/CEN plasmid) also affects yields of heterologous proteins.

The availability of yeast promoters which are efficient in directing expression of a heterologous gene and whose activity can be regulated would be desirable.

Disclosure of the Invention

The present invention is based on the identification and isolation of two genes which are required for efficient cell fusion during yeast conjugation. Transcription of these two genes has been shown to be greatly induced by the presence of the appropriate mating pheromone. Expression of a gene of interest placed under the control of the promoter of either of these two genes has also been shown to be similarly induced by the presence of the appropriate mating pheromone. When the

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promoter-gene of interest combination is present on a high-copy vector in yeast cells stimulated by the appropriate mating pheromone, expression of the fusion product is greatly increased.

05 According to the present invention, DNA encoding a protein of interest is placed under the control of a yeast (e.g., S. cerevisiae) promoter whose transcription is induced by the appropriate mating pheromone. Induction of the yeast promoter occurs as a result of
10 stimulation by the appropriate mating pheromone.

 According to the present invention, DNA encoding the protein of interest (DNA of interest) is fused or linked to the pheromone-inducible yeast promoter, using known techniques; the promoter-DNA of interest fusion is
15 inserted onto an appropriate vector; vectors containing the fusion are introduced into yeast cells (e.g., by transformation); and induction of transcription of the promoter is stimulated by the appropriate mating pheromone (i.e., alpha factor for a cells, a factor for
20 alpha cells). Induction of the yeast promoter in this manner also results in expression of the DNA of interest which is under its control, resulting in production of the protein encoded by the DNA of interest. If the promoter-DNA of interest fusion is inserted onto a high
25 copy vector (such as a high copy two micron vector), stimulation by the appropriate mating pheromone results in a significant increase in induction of the promoter-DNA of interest fusion and production of the encoded protein in substantial quantities. Thus, it is
30 possible to clone DNA (i.e., an entire gene or a gene segment) encoding a protein or polypeptide in yeast, with expression of the DNA being controlled by the inducible

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promoter; the promoter's activity is, in turn, determined by the presence or absence of stimulation by the appropriate mating pheromone.

05 In one embodiment, a DNA fragment from either the FUS1 gene or the FUS2 gene of S. cerevisiae, which includes the FUS1 promoter or the FUS2 promoter, respectively, is fused or linked, using techniques known to those skilled in the art, to DNA encoding a protein of interest. The DNA fragment of the FUS1 or FUS2 gene is a
10 5' segment or N terminal moiety. The yeast DNA fragment - DNA of interest fusion is inserted into a vector and the vector introduced into wild-type yeast cells. Upon stimulation of the yeast cells by the appropriate pheromone, induction of transcription of the yeast gene
15 fragment (5' segment) occurs, the DNA of interest is expressed and a fusion protein which includes the FUS1 (or FUS2) -encoded protein sequence and the protein or polypeptide encoded by the DNA of interest is produced.

For example, DNA from the FUS1 gene which includes
20 the FUS1 promoter and sequences encoding approximately the first 254 amino acids of FUS1 is fused to DNA encoding a protein or polypeptide of interest and the resulting combination inserted onto a high copy two micron vector (to produce a FUS1-DNA of interest
25 plasmid). The resulting plasmid is introduced into wild-type a cells, which are incubated with alpha factor. Alpha factor induces a-type cells to respond; induction of the FUS1 promoter leads to induction of transcription of the DNA of interest to which it is fused. The result
30 is expression of the FUS1-DNA of interest fusion protein (i.e., a fusion protein which is the FUS1-encoded

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protein and the protein encoded by the DNA of interest) at substantial levels.

DNA encompassing the promoter from either the FUS1 gene or the FUS2 gene can be fused to DNA of interest, incorporated into an appropriate vector and introduced into yeast cells, which are incubated with the appropriate mating pheromone. Incubation in this manner causes induction of transcription of the fusion which includes the FUS1 promoter or the FUS2 promoter and DNA of interest and expression of the DNA, resulting in production of the encoded protein or polypeptide.

This approach can be used to produce proteins of interest in substantial amounts in yeast cells. The method of the present invention is particularly valuable because yeast cells containing the fusion can be grown up (cultured) passively and, after sufficient quantities of cells are produced, induced to express the fusion protein (containing the protein of interest) in substantial quantities. This is an important advantage because many foreign proteins are toxic to yeast cells.

Brief Description of the Drawings

Figure 1 is a schematic representation of chromosome III of the yeast Saccharomyces cerevisiae, on which the BIK-FUS1 region adjacent to HIS4 is shown.

Figure 2 shows results of Northern hybridizations of total RNA isolated from yeast strains treated with alpha-factor or solvent minus alpha-factor.

Figure 3 are immunofluorescence micrographs of alpha-factor-induced spheroplasts fixed in formaldehyde and incubated with anti-beta-galactosidase antibody and FITC-conjugated anti-mouse Ig.

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Figure 4 presents the nucleotide sequence of the BIK1-FUS1 region. Position 1 corresponds to position -245 of the HIS4 gene.

05 Figure 5 presents the nucleotide sequence of the FUS2 gene and the deduced amino acid sequence. It includes approximately 500 nucleotides upstream and, thus, encompasses the hexameric repeats thought to be required for regulation.

Detailed Description of the Invention

10 The yeast S. cerevisiae can exist in a diploid or a haploid state; in the former, it has 17 pairs of chromosomes and, in the latter, a single set of chromosomes (one copy of each of the 17 chromosomes). Under favorable conditions, yeast cells exist in the
15 diploid state and multiply rapidly. Multiplication occurs through mitosis, the process by which a diploid cell duplicates its chromosome pairs and divides into a mother cell and a bud, each of which has two copies of every chromosome.

20 When conditions are unfavorable, yeast cells sporulate; cells stop dividing and undergo meiosis, with the result that they duplicate their chromosome pairs and divide into four cells, each having a set of chromosomes. These haploid cells are of either the alpha type or the a
25 type. They can reproduce by mitosis, but produce only haploids as a result. They can enter the diploid life cycle by conjugation, during which an alpha cell and an a cell fuse to form a diploid cell.

30 Conjugation in S. cerevisiae involves the fusion of haploid cells of opposite mating type, followed by the fusion of nuclei to form a diploid. The zygote formed by

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this process buds off diploid cells capable of vegetative growth. Formation of the zygote requires the coordination of two processes--cell fusion and nuclear fusion. Both processes are initiated by mating
05 pheromones: a cells produce a-factor, to which alpha cells specifically respond, and alpha cells produce alpha-factor, to which a cells specifically respond. Duntze, W.D. et al., European Journal of Biochemistry, 35:357-365 (1973); Wilkinson, L.E. and J.R. Pringle,
10 Experimental Cell Research, 89:175-187 (1974).

Cells stimulated by the appropriate mating pheromone produce surface agglutinins (resulting in extensive clumping of conjugating cultures), arrest their cell-cycle at the G1 stage, and elongate to form a
15 discernible tip (a process dubbed "shmooing"). When the appropriate partners have achieved contact, presumably at the shmoo tip, the cells rapidly fuse. Cell fusion requires the degradation and/or reorganization of the cell wall and the fusion of the two plasma membranes.
20 The nuclei subsequently fuse within the dumbbell-shaped zygote formed and the resultant diploid nucleus begins a series of division cycles, each of which yields a new diploid nucleus that enters an emerging bud. Nuclear fusion is not a passive process; like cell fusion, it
25 requires potentiation by the mating factors. Curran, B.P.G. and B.L.A. Carter, Current Genetics, 10:943-945 (1986); Rose, M.D., et al., Molecular and Cellular Biology, 6:3490-3497 (1986).

Two genes involved in the initial zygote formation
30 (cell surface reorganization leading to cytoplasmic fusion) have been identified. They have been designated FUS1 and FUS2. Cloning of FUS1 and FUS2 revealed that

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they share some functional homology. That is, FUS1 on a high copy plasmid can partially suppress a FUS2 mutant; the opposite is also true. FUS1 is essentially unexpressed in vegetative cells, but is strongly induced by incubation of haploid cells with the appropriate mating pheromone. When a cells are incubated with alpha factor, transcription of FUS1 and FUS2 is strongly induced. The same is true of FUS1 when alpha cells are induced by a factor. Mutations in these genes block the conjugation process at a point following cell contact, preventing cytoplasmic fusion and, as a result, nuclear fusion. Genes containing these mutations have been designated fus, for cell fusion defective.

The expression of these FUS genes has been shown to be activated by mating pheromones, an induction that leads to the appearance of the gene product at the tip of the shmoo. This localization suggests that the defect observed in matings between mutants is a direct result of the absence of the gene product, rather than a symptom of some general metabolic defect. Investigation of the FUS1 gene has shown that it is not expressed in vegetative haploid a and alpha cells and diploid a/alpha cells and achieves high levels only in the presence of mating pheromones, suggesting that it functions exclusively during the actual conjugation process.

Induction of the level of FUS1 mRNA was assessed to determine whether its expression is altered during conjugation. Assessment of FUS1 transcription is described in Example 1.

Similarly, when cells containing the FUS1 gene or a portion including the FUS1 promoter fused to DNA of interest are exposed to the appropriate mating pheromone

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(alpha factor), transcription of the FUS1 promoter is greatly induced, and leads to induction of transcription of the DNA which is under the control of the FUS1 promoter. Pheromone induction has been demonstrated by expression of a fusion protein encoded by a FUS1-LACZ fusion which has two components: a FUS1 fragment including the FUS1 promoter region and sequences encoding approximately the first 254 amino acids of FUS1 and the gene encoding beta-galactosidase. Exposure of wild-type a cells containing the fusion on a high-copy 2 micron vector resulted in at least 1000-fold induction of beta-galactosidase. When single-copy derivatives of this fusion were used, an equivalent induction ratio was observed. Pheromone induction has also been demonstrated by expression of a fusion protein encoded by a FUS1-SUC2 fusion, which includes a FUS1 fragment and the gene encoding invertase which lacks the normal signal sequence for invertase.

As a result of the work described herein, two highly inducible yeast promoters have been identified and can be used for achieving substantial levels of expression for a DNA sequence or gene of interest in yeast. The DNA sequence or gene of interest can encode a polypeptide or protein of interest (i.e., one whose expression is desired). Proteins and polypeptides of interest include those not normally expressed in yeast cells (i.e., heterologous proteins), as well as proteins normally expressed in yeast cells but at lower levels than can be achieved through use of the method of the present invention. As used herein, the term protein is meant to include proteins and polypeptides.

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Recombinant DNA techniques known to those in the art are used to obtain the necessary genetic material and to introduce it into yeast cells, which are subsequently cloned. Maniatis, T., et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).

The DNA encoding the protein or polypeptide of interest can be isolated by obtaining mRNA of the desired gene or gene segment. For example, double-stranded DNA can be produced from the mRNA by conventional means. First, a complementary copy of the DNA is made from the mRNA, using conventional RNA technology; the RNA is subsequently removed by breaking the strands, using art-recognized methods. The cDNA is then made double-stranded; E. coli DNA polymerase I can be used for this step.

Synthetic linkers can be added to both ends of the double-stranded DNA (e.g., by using HindIII or EcoRI synthetic oligonucleotide linkers). cDNA having linkers at both ends is introduced into the unique site of a plasmid, which has been cut with the appropriate restriction enzyme. DNA having linkers at both ends can, alternatively, be introduced into a virus or cosmid. For example, pBR322, pM89 or lambdagtWES can be used for this purpose.

Transformation of bacteria with the ligation products will result in transformation of only a fraction of the cells. Of those cells which are transformed, only a portion will contain the recombinant plasmid. Isolation of bacteria containing the initial plasmid and the recombinant plasmid can be accomplished if the initial vector contains a selectable genetic marker, such

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as drug resistance (e.g., antibiotic resistance). When grown on media on which only those cells containing the selectable marker can survive (e.g., on ampicillin-containing media in the case of an ampicillin resistance marker), bacteria containing the initial plasmid and the recombinant plasmid will be the only cells to survive. A further step will be necessary to isolate cells containing the recombinant plasmid (unless the DNA of interest confers a distinguishable phenotype on cells in which it occurs). This can be done, for example, by isolating and separating DNA from transformed cells and analyzing the DNA to identify cells containing the recombinant plasmids. This can be done, for example, by electrophoresis or sequence analysis.

After the recombinant plasmid is cloned, isolated and identified, bacterial cells in which it is included can be grown, resulting in increased numbers of the recombinant plasmid.

The recombinant DNA molecule can be introduced into yeast cells, using conventional procedures. Ito, H. *et al.*, Journal of Bacteriology, 153:163-168 (1983). That cells contain the recombinant DNA molecule can be verified, both by genetic and hybridization techniques.

Host cells (e.g., S. cerevisiae) containing the recombinant DNA molecule are then cultured (in standard media and under standard conditions); once grown, yeast cells are stimulated by the appropriate mating pheromone. This results in induction of transcription of the yeast gene fragment and expression of the protein or polypeptide of interest in substantial quantities. The protein or polypeptide is produced as part of a fusion protein, which includes the FUS1 (or FUS2) -encoded

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protein and the protein or polypeptide encoded by the DNA of interest. The protein or polypeptide is subsequently removed or released from the fusion protein; this can be done, for example, by including a cassette which encodes the invertase signal sequence. This results in secretion of the fusion protein, followed by removal of the signal sequence.

Vectors containing the pheromone-inducible yeast promoter and DNA of interest can be constructed. For example, a recombinant plasmid can be constructed in vitro, using known techniques. One example of such a plasmid includes: the FUS1 promoter, the initiation region of FUS1, and the gene of interest; optionally it can also contain the FUS1 signal peptide. Alternatively, a plasmid can include a 5' segment or an N terminal moiety of the FUS1 gene which also encompasses DNA encoding approximately the first 254 amino acids; a gene of interest; a selectable marker (e.g., ura3) in yeast; a 2 micron autonomously replicating sequence (ARS); a selectable marker (e.g., amp^r) in bacteria and a replication origin for producing the plasmid in E. coli. An example of an appropriate plasmid into which the FUS1 DNA fragment can be inserted is YEp24.

In one embodiment of the method of the present invention for expressing proteins or polypeptides of interest in yeast, a DNA fragment from either the FUS1 gene or the FUS2 gene, which includes the FUS1 or the FUS2 promoter, respectively, is fused or linked to DNA or a gene encoding the polypeptide or protein (DNA of interest). The yeast DNA fragment can include nucleotide sequences in addition to those which make up the promoter sequence and, in one embodiment, also includes sequences

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encoding approximately the first 254 amino acids of FUS1.
The resulting promoter-DNA of interest fusion is
incorporated into a plasmid, using conventional methods.
The DNA which includes the yeast promoter and additional
05 sequences and the DNA encoding the protein of interest
can be produced through genetic engineering techniques
(e.g., by cloning), can be synthesized mechanically or
can be DNA isolated from yeast cells.

The FUS1 or FUS2 promoter will generally be fused or
10 linked to the DNA or gene (i.e., with no intervening
nucleotide sequences other than those which might be
needed for joining the two components). However, the
promoter and the DNA or gene can be separated by
intervening sequences of any length, provided the DNA or
15 gene is under the control of the promoter.

The FUS1-gene of interest fusion or the FUS2-gene of
interest fusion is inserted on an appropriate (i.e., high
copy) vector, which is introduced into yeast cells by
techniques known to those in the art. The vector can
20 also include a DNA sequence encoding a characteristic,
such as ability or inability to metabolize a particular
nutrient or drug resistance, which makes it possible to
select cells into which the vector (and, thus, the
promoter-DNA of interest fusion) has been introduced and
25 in which the DNA it contains is being expressed. Cells
containing the vector are selected (e.g., by culturing on
media containing a substance or lacking a nutrient whose
presence or absence determines cells' ability to survive)
and, after the cells have grown, exposed to the
30 appropriate mating pheromone.

Yeast cells containing the vector are exposed
to/stimulated by the appropriate mating pheromone by the

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addition of essentially pure (either synthetic or naturally occurring) mating pheromone to the culture medium in which the transformed cells are grown, by the addition of a crude preparation of the appropriate mating
05 pheromone, or by culturing with yeast cells of the opposite mating type (i.e., culturing a cells containing the vector with alpha cells producing alpha factor).

In one embodiment of a recombinant plasmid which can be used in the method of the present invention, the
10 following components are included: a) a fragment of a plasmid, such as pBR322, which includes the plasmid DNA replication origin, which makes it possible to propagate DNA in E. coli; b) DNA encoding a selectable genetic marker (e.g., a drug resistance gene, such as amp^r , which
15 makes it possible to select bacteria (E. coli) containing the recombinant plasmid; c) a fragment of the yeast 2 micron autonomously replicating sequence (ARS); d) a DNA fragment encoding a selectable genetic marker (e.g., a drug resistance gene, such as amp^r , ura3), which makes it
20 possible to select yeast containing the recombinant plasmid; e) a fragment of yeast genomic DNA which includes the FUS1 promoter and sequences encoding approximately the first 254 amino acids of FUS1 and makes induction as a result of stimulation by the appropriate
25 mating factor possible; and f) DNA encoding a protein or polypeptide not normally produced in substantial quantities in yeast cells. The latter component is positioned in the construct so as to be under the optimal control of the FUS1 promoter; induction of the FUS1
30 promoter leads to induction of transcription of the DNA of interest.

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The promoters and the method of the present invention, can be used to express a protein of interest, such as tpa, kpa, calf renin and bovine growth hormone. The genes encoding each of these proteins can be the naturally-occurring gene or DNA encoding the protein can be synthesized. For example, as described above, the FUS1 promoter and the first 254 amino acids of FUS1 are fused, using known techniques, to the gene of interest. Maniatis, T. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). They are inserted onto a 2 micron high copy plasmid and introduced into a cells, again using known methods. a cells containing the plasmid and the FUS1 promoter-gene of interest fusion are stimulated by addition of growing alpha cells. In this way, the a cells are stimulated by the alpha factor and induction of the FUS1 promoter occurs and leads to induction of the DNA encoding the protein of interest.

In a method for obtaining expression of a protein or polypeptide of interest in yeast according to the present invention, a portion of either the FUS1 or the FUS2 gene which includes the respective promoter is fused to DNA encoding the protein or polypeptide. This is done in such a way that the DNA of interest is placed under the control of the FUS1 or FUS2 promoter; preferably, it is placed under the optimal control of the promoter. The fusion produced is introduced into cells, in which the genetic information is retained and passed on to subsequent generations. Construction of a yeast strain having these characteristics (a pheromone-inducible promoter linked to DNA encoding a protein or polypeptide of interest) is useful because such cells can be used

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(e.g., commercially) to produce the encoded products. Large-scale yeast fermentation methods are well developed and S. cerevisiae has no disadvantageous characteristics (e.g., toxicity).

05 In a further embodiment of the present invention, DNA encoding protein or polypeptide of interest is introduced into yeast cells through the use of retroviral vectors containing the pheromone-inducible promoter, DNA
10 of interest and other components necessary for expression of the encoded protein.

 The host cell, in which the pheromone-inducible promoter-DNA of interest fusion is expressed, will generally be the yeast strain S. cerevisiae. S. cerevisiae has distinct advantages, as explained
15 previously. However, other yeast cells which can be transformed with the promoter-DNA of interest fusion can also be used.

 Cultivation of yeast can be carried out under well-standardized conditions; this is the case with yeast
20 cells into which the promoter-DNA of interest fusion is introduced. For example, yeast cells can be grown on commonly used laboratory media, such as yeast nitrogen base (YNB, Difco). Stimulation of yeast cells to induce transcription of the promoter and DNA of interest can be
25 carried out in this media, with the addition of mating factor, or of yeast cells of the opposite mating type (which will produce the pheromone necessary to stimulate induction). In one embodiment of the present invention, approximately 8 micrograms (5 micromolar) of mating
30 factor is included per milliliter of culture media. If yeast cells containing the FUS1 DNA fragment - DNA of interest fusion are cultured with yeast cells of the

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opposite mating type, an equal volume of each of the two types of yeasts is used. That is, cells of equal density and at the same stage of growth are cultured together; in this case, both types of cells are growing exponentially.

05 It is evident from the above description that the pheromone-inducible yeast promoter can be introduced into yeast cells, along with DNA which encodes one or more proteins of interest and transcription of which is under control of the promoter, and used to produce proteins
10 having a wide variety of applications (e.g., as drugs, enzymes, constituents of foods and beverages).

The present invention will now be illustrated with the following examples, which are not to be seen as limiting in any way.

15 Example 1 Induction of FUS1 and FUS2 by mating pheromones.

Induction of the FUS1 and the FUS2 genes was assessed as follows: For induction by alpha-factor, cells were grown to Klett 40 in YPD or SC-ura (for
20 selection of plasmids) which had been titrated to pH 4 with hydrochloric acid. Alpha-factor (Sigma Chemical Company) was added to a concentration of 5 micromolar (uM) (1:100 dilution of a 0.5 mM solution in methanol), or methanol was added to a concentration of 1%. Cells
25 were grown for two additional hours at 30°C, at which time more than 90% of the alpha-factor treated population had arrested as shmoos or unbudded cells.

For induction by a-factor, cells were pregrown in untitrated SC-ura to Klett 40-50, and then pelleted and
30 resuspended in an equal volume of YPD media in which JY132 cells (MATa) or EEX8 cells (a/alpha) were growing

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at a similar density, or in an equal volume of unconditioned YPD. The cells were then grown for two more hours at 30°C.

Northern analysis. Total RNA was isolated from cells
05 treated for 2 hours with alpha-factor or from control
cells using the method of Carlson and Botstein. Carlson,
M. and D. Botstein, Cell, 28:145-154 (1982). The
isolated total RNA was separated by electrophoresis
through a 1.5% agarose denaturing gel, transferred to the
10 nylon membrane GeneScreen Plus according to the
manufacturer's instructions (New England Nuclear Research
Products, Boston), and hybridized, either with labelled
RNA obtained from in vitro SP6 transcription (Promega
Biotec, Madison, WI) or with DNA labelled by nick
15 translation. Maniatis, T. et al., Molecular Cloning: A
Laboratory Manual, Cold Spring Harbor Laboratory, Cold
Spring Harbor, N.Y. (1982). To obtain a single-stranded
probe specific for FUS1, a fragment internal to the gene
was inserted into an SP6 vector in the proper orientation
20 and transcribed in vitro in the presence of labelled
nucleotide. The RNA probe extended from nucleotide 3635
to 4030 (see Figure 4) of the FUS1 gene. The DNA probe
consisted of the BIK1-FUS1 HindIII fragment inserted into
the URA3 vector YIp5. Figure 2 shows the resultant
25 autoradiogram, as well as a duplicate filter probed with
the 6 kb HindIII fragment containing the entire FUS1-BIK1
region inserted into the URA3 vector YIp5. The addition
of the alpha-factor pheromone to a/a cells, but not to
isogenic a/alpha cells, caused an induction of a 1.6 kb
30 message not observed in cells that were not exposed to
the pheromone. The Northern blot on the left in Figure 2

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is a control showing that an approximately equivalent amount of RNA was loaded onto each lane. The FUS1 gene is a prototype for the FUS2 gene. The same procedure was followed using the FUS2 gene and similar results were
05 obtained.

Beta-galactosidase assays. Cells were permeabilized by vigorous agitation in Z-buffer supplemented with 0.0075% SDS and 60 ul/ml chloroform, and the assays were performed essentially as described by Miller. Miller,
10 J.H., Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1972). Units of beta-galactosidase activity are expressed by the formula $(1000 \times \text{O.D.}_{420} \text{ of centrifuged reaction mixture}) /$
($\text{O.D.}_{600} \text{ of culture} \times \text{volume of culture} \times \text{minutes of}$
15 assay).

Example 2 Construction and expression of
FUS1-heterologous DNA.

The pheromone effect described in Example 1 was analyzed further by the construction of a FUS1-LACZ
20 fusion that had the promoter region of FUS1 and sequences which encode the first 254 amino acids of FUS1 fused to beta-galactosidase. Addition of alpha-factor to wild type a cells containing this fusion on a high copy micron
vector led to at least a 1000-fold induction of
25 beta-galactosidase, as shown in Table 1.

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Table 1. Induction of FUS1-LACZ by mating pheromone.

	Uninduced	Induced*
JY132(pSB234) (<u>MATa</u>)	0.5	740
JY133(pSB234) (<u>MATalpha</u>)	4.7	650

05 pSB234 is a 2 micron-URA3-based plasmid which encodes the
FUS1-LACZ gene product; the fusion contained the first
254 amino acids of FUS1 fused to beta-galactosidase.
Units of activity were determined as described in Example
1. An equivalent induction ratio was also seen for
10 single copy derivatives of this fusion.

Incubation of alpha cells containing the same
FUS1-LACZ plasmid with wild type a cells (a source of
a-factor) also caused substantial induction; the
uninduced level in alpha cells appeared to be
15 significantly higher than in a cells. The same procedure
was followed using the FUS2 gene and similar results were
obtained.

Localization of FUS1-LACZ. The cellular location of the
FUS1 gene product was determined by immunofluorescence
20 microscopy on pheromone-treated cells containing the LacZ
fusion. Kilmartin, J.V. and A.E.M. Adams, Journal of
Cell Biology, 98:922-933 (1984). A mouse monoclonal
alpha-beta-galactosidase antibody (provided by T. Mason)
was used as a probe. It was visualized using a
25 FITC-conjugated goat anti-mouse antibody (Boehringer
Mannheim Biochemicals, Indianapolis, IN). Figure 3 shows
a series of micrographs from such cells, with a CYC1-LACZ

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fusion in similarly treated cells acting as a control fusion that should be cytoplasmic. In a large fraction of the cells, the FUS1-LacZ protein appears to be located exclusively at the tip of the shmoo. The FUS2-LacZ protein was localized using similar techniques. It appears to be located within the schmoo, but not at the tip of the schmoo.

Example 3 Determination of the sequence of the FUS1-BIK1 region.

10 The 6 kb HindIII fragment containing FUS1 and BIK1 was inserted into the unique HindIII site of YCp50, in the orientation bla-BIK1-FUS1-tet. Farabaugh, P.J. and G. R. Fink, Nature, 286:352-356 (1980). The unique BamHI site of this plasmid was subsequently destroyed by
15 filling in the ends with Klenow fragment and religation of the blunt ends, generating pJef423. All complementation analyses were performed using derivatives of this plasmid.

Linker insertion mutagenesis was performed by DNase
20 I nicking, micrococcal nuclease digestion followed by digestion with S1 nuclease, and ligation of BamHI linkers, essentially as described by Shortle. Shortle, D., Gene, 22:181-189 (1983). The sequence of the BIK1-FUS1 region was obtained by inserting various
25 restriction fragments, including those obtained from the BamHI insertions, into the M13 phage derivatives mp18 and mp19, and sequencing by the dideoxy chain termination method. Biggin, M.D. et al., Proceedings of the National Academy of Sciences, U.S.A., 80:3963-3965 (1983); Sanger,
30 F. et al., Proceedings of the National Academy of Sciences, U.S.A., 74:5463-5467 (1977).

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The precise structure of the cloned FUS1 gene was determined by dideoxy sequencing of a collection of M13 mp18 and M13 mp19 phage containing different segments of the entire region, including BIK1 sequences. The sequence is presented in Figure 4. Except for small stretches of the intervening open reading frame, sequencing was carried out on both strands of the DNA. The sequence differs at four positions between nucleotides 519 and 540 from that previously determined. Donahue, T.F. *et al.*, Gene, 18:47-59 (1982). In three of these positions, an extra T was determined to occur and at one position, a T rather than an A was found. The sequence of this region is: GTAGCTGTTTCATTCTCAGCGTC. The BIK1 coding region extends to within 14 bases of the first upstream repeat (TGACTC) of the HIS4 gene.

The FUS1 and FUS2 genes were isolated as described below. For FUS1, this was done as follows: A yeast strain (L1052) containing a large deletion (Δ 453) extending from HIS4 to LEU2 was transformed with various derivatives of pJef423 harboring linker insertions. These transformants were assayed by the replica-plating test for their ability to mate successfully with a Δ 453 tester lawn, L1546. Large linker-associated deletions (e.g. 550, 552; Figure 1) in the HindIII fragment completely abolished the ability of the plasmid to complement the Δ 453 defect; however, less extensive linker-associated deletions (e.g. 514, 543) and "simple" linker insertions (less than 50 bp deleted; 518, 483) only partially compromised the complementing activity of the HindIII insert. From this mutational analysis, a portion of which is presented in Figure 1, a map was constructed which contains two distinct regions of

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complementing activity; either region alone is able to restore only partial mating competence to the $\Delta 453$ recipient.

05 With the exception of 565, 566, 514, and $\Delta 136$, all of the mutations shown in Figure 1 were integrated into the chromosome of wild type cells (JY132 and JY133). The linker insertions were moved to URA3 integrating vectors, and the resultant plasmids were directed to integrate at chromosome III by cutting at the unique KpnI site before
10 transformation. Orr-Weaver, T. L. et al., Proceedings of the National Academy of Sciences, U.S.A., 78:6354-6358 (1981).

The recipient strains harbored the his4-34 mutation, which lies within the region of his4 that is duplicated
15 upon integration of these plasmids. Therefore, His⁺ recombinants were selected for, in order to obtain the appropriate "loop-out" excision event. Those His⁺ derivatives which had simultaneously become Ura⁻ harbored the appropriate linker insertion (or frameshifted
20 restriction site) and no plasmid sequences. Complementation tests using these strains transformed with the original set of pJef423 derivatives confirmed the idea that two genes required for efficient mating (FUS1 and FUS2) reside on the HindIII fragment upstream
25 from HIS4.

The fus2-1 mutation was uncovered in a cross between C52a and JY145 (fus1-483). Several tetrads from this cross produced one ascospore which, when mated to $\Delta 453$ lawns, displayed a drastic defect as compared to the
30 response with wild type lawns. (fus1 alone displays only a partial defect when mated to $\Delta 453$). These segregants were outcrossed to wild type strains, and the segregation

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of wild type (good mating with all lawns), partially defective (poor mating with mutant parent lawns, but partial mating with fus1 lawns), and completely defective (poor mating with fus1 lawns and with mutant parent lawns) phenotypes among the spore clones suggested the presence of two unlinked fus mutations in the mutant parent. Single fus mutants mate poorly with double mutants, but partially with single mutants, and conversely, double mutants mate poorly with single mutants and double mutants (but mate well with wild type). The mating type of mutants does not affect their phenotype as assayed by replica plating.

The fus1 mutation was identified in strains carrying a deletion extending from HIS4 to LEU2 (Figure 1). This large deletion, designated Δ 453, had no obvious effect on the vegetative growth of the cells, but subsequent genetic manipulations revealed that Δ 453 strains form diploids at greatly reduced rates with strains carrying the same deletion, but at relatively normal rates with wild type strains. A centromere plasmid bearing the 6 kb HindIII fragment beginning in HIS4 and extending towards LEU2, subcloned from a 15 kb HIS4 BamHI-EcoRI fragment (isolated by "eviction"), was found to restore normal mating to a Δ 453 strain (L1052), suggesting that this segment contains the mating functions missing from Δ 453. Donahue, T.F. *et al.*, Cell, 32:89-98 (1983).

Extensive deletion analysis and random linker insertion mutagenesis (a subset of which is presented in Figure 1) reveal that the phenotype of Δ 453 results from deletion of two separate genes, both of which are located on the HindIII fragment. The genes were further defined by introducing the linker insertions into the chromosome

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of wild type cells and analyzing the behavior of the resulting strains in crosses. The linker insertions fall into two groups based on complementation tests. Insertions in one of these groups leads to a block in cellular fusion in crosses between members of the same group. The gene defined by this complementation group is called fus1. Mutants in the second group mate normally with wild type and fus1 mutants; however, in crosses with members of the same complementation group, the cells fuse normally but their nuclei fail to fuse. The gene defined by this complementation group is called bik1 (bilateral karyogamy defect). These experiments showed that the mating defect of $\Delta 453$ results from the deletion of two genes: BIK1 and FUS1, each of which has its own unique function (see Figure 1).

Isolation of FUS2 was carried out as follows: The fus2 mutation was uncovered in crosses of fus1 by strain C52a, obtained from C. Nombela. The phenotype of fus1 mutants is rather leaky; many pairs in a fus1 x fus1 cross fuse their nuclei despite the abnormal bridge between the pair. In crosses of JY146 (alpha fus1-483 ura3-52 leu2-3, 112) by C52a (a exb1-1) several ascospore segregants were obtained which displayed a much more severe fusion defect than that of the fus1 mutant. These segregants were shown by genetic analysis to be double mutants, fus1 fus2. The fus2 mutation was apparently present in C52a, although it is not linked to the exb1-1 mutation described by Santos et al. (as assayed by the methylumbelliferyl-beta-D-glucoside overlay technique). As shown by subsequent tests, fus2 has a phenotype similar to that of fus1.

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To clone the FUS2 gene, the severe mating defect of a fus2 mutant when mated with a fus1 fus2 double mutant was used. A FUS⁺ strain can readily mate with a fus double mutant in the replica plating assay and the fus2 defect is recessive; as a result, cloning by complementation is straightforward. A MATa fus2 strain (JY306) was used as a recipient in transformation with the Yep24 library of Carlson and Botstein. Carlson, M. and D. Botstein, Cell, 28:145-154 (1982). A clone (pSB257) was identified (out of about 2000 screened) which restored normal mating function to fus2 when mated to fus1 fus2 (JY217). Genetic analysis demonstrates that the 9 kb DNA segment isolated corresponds to FUS2: crosses between FUS⁺ strains and a fus2 strain harboring a FUS2⁺ -URA3⁺ plasmid integrated at fus2 yielded 19 out of 21 tetrads which contain 4 FUS⁺ spores. One-step gene disruption by the substitution of an internal 1.1 kb HindIII fragment with the URA3 gene resulted in a phenotype identical to that of fus2-1: a gross mating defect with fus1 fus2 strains, but only slightly reduced diploid formation with either fus2-1 or fus2::URA3 strains.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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CLAIMS

1. A DNA fragment comprising a yeast promoter linked to DNA encoding at least one polypeptide not normally expressed at substantial levels in yeast cells,
05 transcription of the yeast promoter inducible by a yeast mating pheromone, for directing the expression of the polypeptide-encoding DNA in yeast cells.
2. A DNA fragment of Claim 1 wherein the yeast promoter is a FUS1 promoter or a FUS2 promoter.
- 10 3. A DNA fragment of Claim 2 additionally comprising a nucleotide sequence encoding the first 254 amino acids of the FUS1 gene.
4. A DNA fragment of Claim 2 additionally comprising a nucleotide sequence encoding the first 617 amino
15 acids of the FUS2 gene.
5. A DNA fragment of Claim 1 wherein the yeast promoter has the sequence consisting essentially of nucleotides 2550 to 3224 of Figure 4.
- 20 6. A DNA fragment of Claim 1 wherein the yeast promoter has the sequence consisting essentially of nucleotides 1 to 402 of Figure 5.
7. A DNA fragment of Claim 3 having the sequence consisting essentially of nucleotides 2550 to 3987 of Figure 4.

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8. A DNA fragment of Claim 4 having the sequence consisting essentially of nucleotides 1 to 2253 of Figure 5.
- 05 9. Isolated DNA having the sequence consisting essentially of nucleotides 2550 to 3224 of Figure 4.
- 10 10. Isolated DNA having the sequence consisting essentially of nucleotides 2550 to 3987 of Figure 4.
11. Isolated DNA having the sequence consisting essentially of nucleotides 1 to 402 of Figure 5.
- 10 12. Isolated DNA having the sequence consisting essentially of nucleotides 1 to 2253 of Figure 5.
- 15 13. A yeast promoter, inducible by the appropriate mating pheromone, linked to DNA encoding a protein or a polypeptide not normally expressed at substantial levels in yeast cells.
14. A yeast promoter of Claim 13 which is selected from the group consisting of the FUS1 promoter inducible by alpha factor and the FUS2 promoter inducible by a factor.
- 20 15. A recombinant DNA sequence which is nucleotides 2550 to 3987 of Figure 4 fused with X, wherein X is DNA encoding a protein or a polypeptide not normally expressed at substantial levels in yeast cells.

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16. A recombinant DNA sequence which is nucleotides 1 to 2253 of Figure 5 fused with X, wherein X is DNA encoding a protein or a polypeptide not normally expressed at substantial levels in yeast cells.
- 05 17. A recombinant DNA sequence which is nucleotides 2550 to 3224 of Figure 4 fused with X, wherein X is DNA encoding a protein or a polypeptide not normally expressed at substantial levels in yeast cells.
- 10 18. A recombinant DNA sequence which is nucleotides 1 to 402 of Figure 5 fused with X, wherein X is DNA encoding a protein or a polypeptide not normally expressed at substantial levels in yeast cells.
19. The recombinant DNA sequence of Claim 15, wherein the polypeptide is beta-galactosidase.
- 15 20. The recombinant DNA sequence of Claim 16, wherein the polypeptide is beta-galactosidase.
21. The recombinant DNA sequence of Claim 17, wherein the polypeptide is beta-galactosidase.
22. The recombinant DNA sequence of Claim 18, wherein
20 the polypeptide is beta-galactosidase.
23. A method of expressing a protein or a polypeptide in yeast cells, comprising the steps of:
 - a. introducing into yeast cells a recombinant vector which contains a DNA fusion, the DNA
25 fusion comprising a pheromone-inducible yeast

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- promoter and DNA encoding a protein or a polypeptide to be expressed; and
- b. stimulating yeast cells containing the recombinant vector with the appropriate mating pheromone.
- 05
24. A method of Claim 23 wherein the recombinant vector is a recombinant two micron yeast vector.
25. A method of Claim 24 wherein the yeast cells are wild type a yeast cells and the mating pheromone is alpha factor.
- 10
26. A method of Claim 24 wherein the yeast cells are wild type alpha yeast cells and the mating pheromone is a factor.
27. A protein or a polypeptide produced by the method of Claim 25, wherein the DNA fusion has the sequence consisting essentially of nucleotides 2550 to 3987 of Figure 4 fused with X, wherein X is DNA encoding a protein or a polypeptide not normally expressed at substantial levels in yeast cells.
- 15
28. A protein or a polypeptide produced by the method of Claim 26, wherein the DNA fusion has the sequence consisting essentially of nucleotides 2550 to 3987 of Figure 4 fused with X, wherein X is DNA encoding a protein or a polypeptide not normally expressed at substantial levels in yeast cells.
- 20
- 25

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- 05 29. A protein or a polypeptide produced by the method of Claim 25, wherein the DNA fusion has the sequence consisting essentially of nucleotides 1 to 2253 of Figure 5 fused with X, wherein X is DNA encoding a protein or a polypeptide not normally expressed at substantial levels in yeast cells.
- 10 30. A protein or a polypeptide produced by the method of Claim 26, wherein the DNA fusion has the sequence consisting essentially of nucleotides 1 to 2253 of Figure 5 fused with X, wherein X is DNA encoding a protein or polypeptide not normally expressed at substantial levels in yeast cells.
- 15 31. A protein or polypeptide produced by the method of Claim 25, wherein the DNA fusion has the sequence consisting essentially of nucleotides 2550 to 3224 of Figure 4 fused with X, wherein X is DNA encoding a protein or a polypeptide not normally expressed at substantial levels in yeast cells.
- 20 32. A protein or polypeptide produced by the method of Claim 26, wherein the DNA fusion has the sequence consisting essentially of nucleotides 2550 to 3224 of Figure 4 fused with X, wherein X is DNA encoding a protein or a polypeptide not normally expressed at substantial levels in yeast cells.
- 25 33. A protein or a polypeptide produced by the method of Claim 25, wherein the DNA fusion has the sequence consisting essentially of nucleotides 1 to 402 of Figure 5 fused with X, wherein X is DNA encoding a

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protein or a polypeptide not normally expressed at substantial levels in yeast cells.

- 05 34. A protein or a polypeptide produced by the method of Claim 26, wherein the DNA fusion has the sequence consisting essentially of nucleotides 1 to 402 of Figure 5 fused with X, wherein X is DNA encoding a protein or a polypeptide not normally expressed at substantial levels in yeast cells.
- 10 35. A recombinant yeast cell containing an expression vector, the expression vector comprising a yeast DNA fragment including a pheromone-inducible promoter fused to DNA encoding a protein or polypeptide of interest, said protein or polypeptide not normally produced in significant amounts in yeast cells.
- 15 36. A recombinant plasmid comprising:
- a. the FUSI promoter;
 - b. DNA encoding approximately the first 254 amino acids of the FUSI gene;
 - c. DNA encoding a protein or a polypeptide of
20 interest, the DNA positioned so as to be under the control of the FUSI promoter;
 - d. DNA encoding a selectable marker in yeast;
 - e. a two micron autonomously replicating sequence;
 - f. DNA encoding a selectable marker in bacteria;
25 and
 - g. a replication origin for producing the plasmid in bacteria.

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37. A recombinant plasmid comprising:
- a. the FUS2 promoter;
 - b. DNA encoding approximately the first 614 amino acids of the FUS2 gene;
 - 05 c. DNA encoding a protein or a polypeptide of interest, the DNA positioned so as to be under the control of the FUS2 promoter;
 - d. DNA encoding a selectable marker in yeast;
 - e. a two micron autonomously replicating sequence;
 - 10 f. DNA encoding a selectable marker in bacteria; and
 - g. a replication origin for producing the plasmid in bacteria.
38. Plasmid YEp24 having inserted therein the
15 recombinant DNA sequence of Claim 15.
39. Plasmid YEp24 having inserted therein the recombinant DNA sequence of Claim 16.
40. Plasmid YEp24 having inserted therein the recombinant DNA sequence of Claim 17.
- 20 41. Plasmid YEp24 having inserted therein the recombinant DNA sequence of Claim 18.

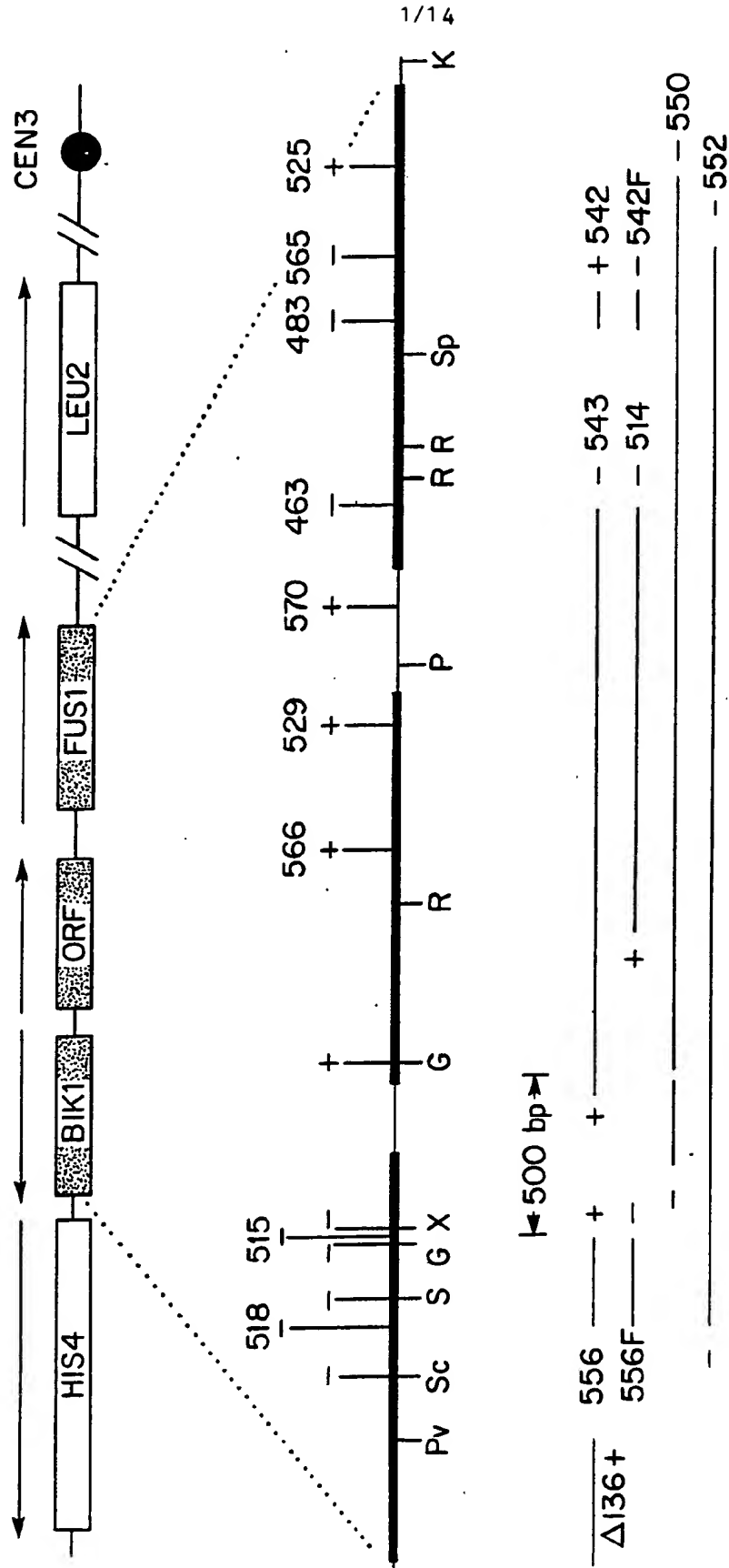


Fig. 1

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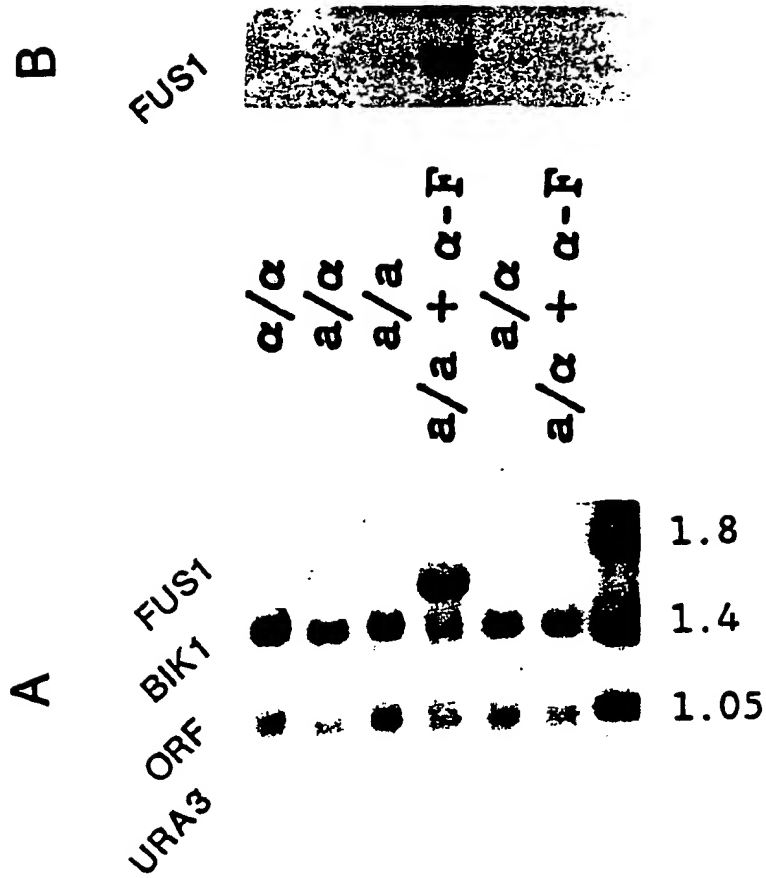
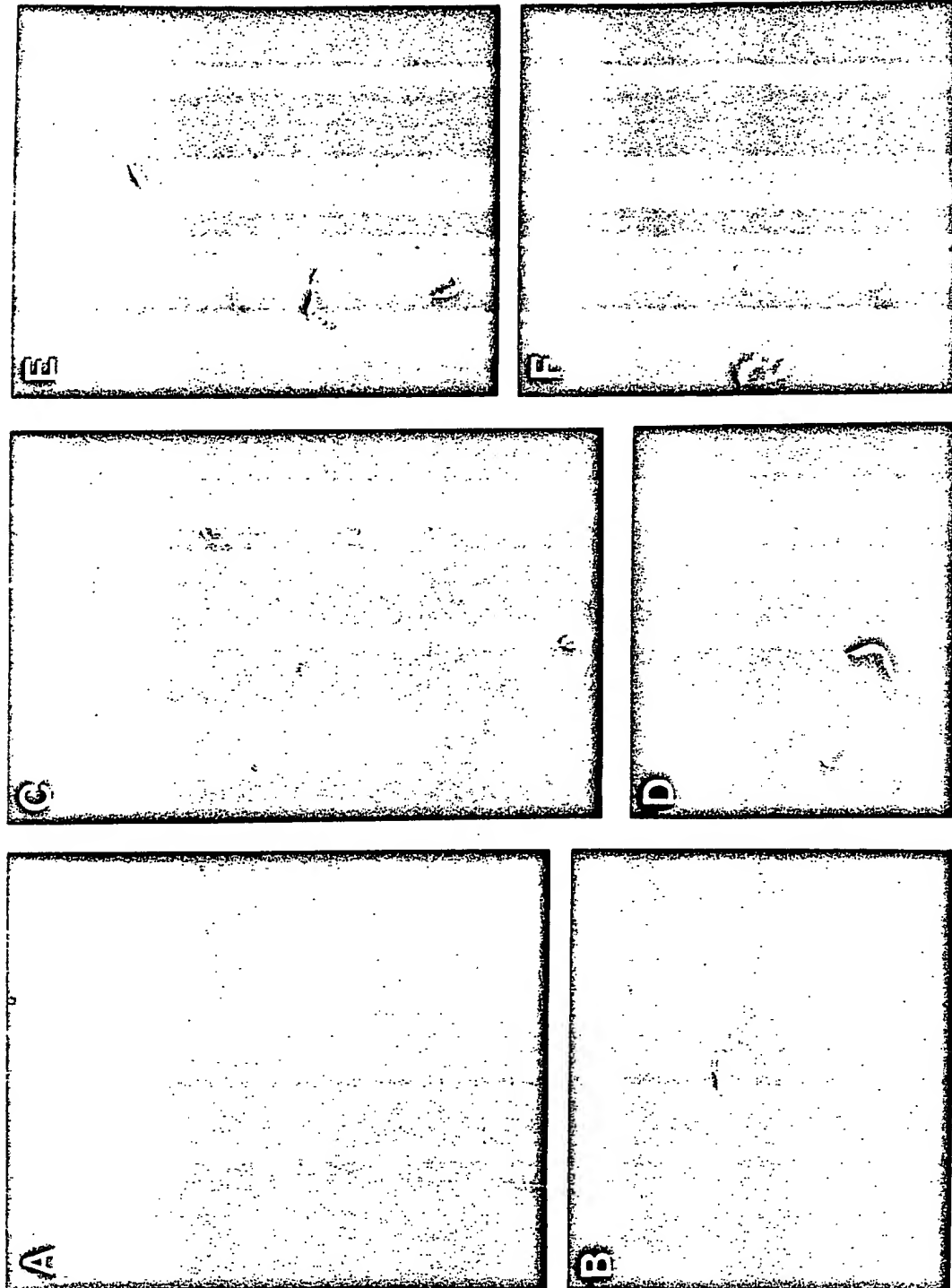


FIG.2

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FIG.3



SUBSTITUTE SHEET

FIG. 4

1 TATL A G A G T C A G T T C G A C T G C C T A G A A G A A C T G C I G G T T G T C A G G A T T G T G A T G G G G C A T T C T G C T G T A T T A T G A C C C A T C G T A T C G C A A T G C T C A C A C C A C T G T T G T C T T C C T G C C G T 120
 A T A A T C T C A G T C A A G C T G A C G G A T C T T C T T G A C G A C C A A C A G T C C T A A C A C T A C C C C G T A A G A C G A C A T A A T A C T G G T A G C A T A G C G T T A C G A G T G T G G T G A C A A C A G A A G G A C G G C A
 EndPhePheGlnGlnAsnAspProAsnHisHisProCysGluAlaThrAsnHisGlyMetThrAspCysHisGluCysTrpGlnGlnArgGlyAlaThr
 121 G G T A T C G A C T G G T G C A G G G G G T C G A A A A T T G G C A A C G A T T C C A C G G C T G T T G C T T G A G C C T G T T C C A C T G T T G A T T T T T T C A T T A G C C T C T T C A A G T T T T T C G T T A A G G A T G C 240
 C C A T A G C T G A C C A C G T C C C C C A G C T T T A A C C G T T G C T A A G T G C C G A C A A C A C G A A C T C G G A C A A G T T G A C A A A C T A A A A A A G T A A T C G G A G A A G T T C A A A A A A G C A A T T C C T A C G
 ThrAspValProAlaProProAspPheIleProLeuSerGluValAlaThrGlnAlaGlnGlnLeuGlnLysIleLysGluAsnAlaGluGluLysLysThrLeuSerAla
 241 C A C C T C T T C C G A T G A G G A A T C T T G T G G T T T T G T C A A A A T A G T T C C T T G C T C A A A T T T T G G T A T T C T T A C T G A G C G A A T C G T T A T G C A T T T T C A A T T G T T C G C G T T C T T T A G C C C A C T T 360
 G T G G A G A G G C T A C T C C T T A G A A C A C C A A A C A G A T T T T A T C A A G G A A C G A G T T T A A A A C C A T A A G A A T A C T G C T T A G C A A T A C G T A A A A G T T A A C A A G C G C A A G A A T C G G G T G A A
 ValGluGluSerSerSerAspGlnProLysThrLeuPheLeuGluLysSerLeuAsnGlnTyrgLulysSerLeuSerAspAsnHisMetLysLeuGlnGluArgGluLysAlaTrpLys
 361 T G T C T T G T G T A C T C A A A T T G G T C T T C T A T G T T G C G T A A T T G T T C C A G C T G T T T T T C A G G A G T T C G A C A T C T T C G T T G G C A C C A G T G G G T T G A T T A T G A G A A A G A T T T C T C T C T T C G T T 480
 A C A G A A C A C A T T G A G T T T A A C C A G A C A T A C A A C G C A T T A A C A A G G T C G A C A A A A A A G T C C T C A A G C T G T A G A A G C A A C C G T G G T C A C C C A C T A A T A C T C T T T C T A A A G A G A G A A G C A A
 ThrLysHisLeuGluPheGlnAspGluIleAsnArgLeuGlnGluLeuGlnLysLysLeuLeuValAspGluAsnAlaGlyThrProGlnAsnHisSerLeuAsnArgGluGluGluAsn
 481 T T C T T T G A T C T C T T C G T A G T T G G C T T A C G A C A G A A G T A G C T G T T C A T T C T C A G C G T C A A A A A C T G C T T T T G T T T G G C T T G C T G C T G C G T T C G A G C T C C A A T T G T T G C T T G A G A T G 600
 A A G A A A C T A G A G A A G C A C A T C A A C C G A A T G C T G T G T T C A T C G A C A A G T A A G A G T C G C A G T T T T T G A C G A A A A C A A C C G A A C G A C A G A C G A A G C T C G A G T T A A C A A C G A A C T C T A C
 GluLysIleGluGluHisLeuGlnSerValValAlaLeuLeuGlnGlnLysGlnLysAlaAspPhePheGlnLysGlnLysAlaGlnGlnArgArgGluLeuGluGlnGlnLysLeuHis

FIG. 4 CONT.

601 GICATCTCTTCTCTCTTCTTGTATTGTGGCTTCATACCTATCAAAAGTCGGTTGCACCTCTTCGAGGACCATTCTTTGGTCATCGAGTAGCCCTTTTGTAGTGTAGTTTCCITTG 720
 CAGATAGAGAAAGAGAGAGAAAGACATAACACCGAAGTATGGATAGTTTTCAGCCAACGTTGAAGAACCTCCTGGTAAGAAACCAGTAGCTCATCGGAAACACATCACATCAACAAAGGAAC
 AspIleGluLysGluArgGluGlnIleThrAlaGluTyrArgAspPheThrProGlnValGluGluValMetArgGlnAspAspLeuLeuArgLysTyrHisLeuGlnLysArgGln
 721 TAGCTTTTCGATGGTCAATTGGGCTTCTCGTAATTCATTCGCTGCTATTGAGGTCAATTCATGTGGCCATTGTCCGGTTTCCAATCGTGGTGGTGTGTGTAGTTCCTTTCT 840
 ATCGAAAGCTACAGTTAACCAGGAGGAGCATTAAGTTAACTTGAAGCGACGATAAATCCAGTAAGTACACCGGTAAAGGTTACAGGCCAAAGGTTACCGACCCACACACACTAATCGGAAAGA
 LeuLysGluIleThrLeuGlnAlaGluArgLeuGluIleThrValGluSerSerAsnLeuAspAsnMetHisGlyAsnAspProLysTrpAspSerThrThrAsnHisAsnAlaLysArg
 841 GTCGTGATGACAGGATAGAGTCGACCTCCATTCTGTCTCTCTGTTATCGTAACCAAAATTCCTTGCTGTTGATGGTGATCCGATGCCCTCCTGCTGCTCCATCGACTGTTGATTACCGCTGCGCG 960
 CAGACTACTGTCTATCTCAGCTGGAGGTAAAGACAGAGACAAATAGCATTTGGTTTAAGAACGACAACTACCACTAGGCTACGGAGGCCAGGTAGCTGACAACTAATGGCGACACGGC
 AspSerSerLeuIleSerAspValGluMetArgAspGluArgAsnAspTyrGlyPheGluGlnGlnHisAspSerAlaGluGlnAspMetSerGlnGlnAsnGlySerHisArg
 961 ACTGGTGATCCGGAACCTCTCATGGGTGGGGATTAGGATCATCCATGGGAGAGAACTGGTTAGTGAGCCTCACAAATAGATCTGTTTTTTGGGTATTGATAGCGGTTCCATTGTCTGT 1080
 TGACCACTAGGCCTTTGAAGAGTACCCACACCCCTAAATCCTAGTAGGTACCTCTCTTGACCAATCACCTCGGAGTGTATCTAGACAAAACCCCACTAATCGCCCAAGGTAAACGCA
 SerThrIleArgPheSerArgMetProThrProSerLysProAspAspMetProSerPheGlnAsnThrLeuArgValIleSerArgAsnLysProIleSerLeuProGluMetThrThr
 1081 TCTTCTCGAGGTTTGGGATATCGATGCCCTTCTCGATCAATGATCGGACTTTTTCGAACTGAATAATAGTCCACTTTGAGGATACCTCGTTTGAAATACTTCTTCCCATGAATGATCC 1200
 AGAAGAGCTCCAAACGCTATAGTACGGAGAGGCTAGTTACTACGCTGAAACCGTTGACTTATTTATCAGGTGAAACCTCCTATGAGGCCAACTTTTATGAAGAGGGGTACTTACTAGG
 ArgArgSerThrGlnSerIleSerAlaLysGluIleLeuSerAlaValLysGlnLeuGlnIlePheLeuGlySerGlnProTyrGluThrGlnPheTyrLysLysGlyMetPheSerGly

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FIG. 4 CONT.

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1201 ATCGTTCTTACCAATGTTGCAAGTAAGTCTACACCAGCAAACATTCCAGCTTTCAGTGTCCACTGGACCCACCGTATTTCAGTTGTCGCGGCCCAAATTTGGGATTGTATGAACATCC 1320
TAGCAAGAAATGGTTACAACCGTTTCATTTCAGATGTGGTCGTTTGTAAAGTCGAAAGCACAGGTGACCTGGGTGCATAAAGTCAACAGGCGCGGGTTTAAACCCATAACATACITTTGTAGG
AspAsnLysGlyIleAsnAlaLeuLeuAspValGlyAlaPheMetGlyAlaLysThrAspValProGlyValTyrLysLeuGlnGlyArgGlyLeuAsnProIleGlnIlePheCysGly
1321 TATCTTTCTTGTATATCCATGGTATTTCAAACGCATACACAGCCAGCCACAGCTCAACGCCITTTACCTTGTCCTTTGATGCCGTGTCGICCAAACGTTTTTGGTGTCCTGGCCA 1440
ATAGAAAGCAAACTATAGATAGGTA
IleLysArgGlnTyrArgAspMet
← BIK 1

1441 ATTGCCCTTCTGAAAAATCTCACTGTCGCGCAACTCATTAAAGATATCCCAAGCAAGCTACACGATAAAGAAAGGAGAAAGTTCATTAAAGAACGTACATATAGCGATACAAACGTATAG 1560

1561 CAAAGATCTGAAATGGATACGGATAAGTTAATCTCAGAGGCTGAGTCTCATTTTTCICAAGGAAACCATGCAGAAAGCTGTTCGGAAGTTGACATCCGACAGCTCAGTCGAAACCCCAATGAC 1680
MetAspThrAspLysLeuIleSerGluAlaGluSerHisPheSerGlnGlyAsnHisAlaGluAlaValAlaLysLeuThrSerAlaAlaGlnSerAsnProAsnAsp

1681 GAGCAAAATGTCAACTATTGAATCATTAAATTCAAAAAATCGCAGGATACGTCATGGACAACCGTAGTGGTAGTGACGCGCTCGCAAGATCGTGTGCTGGTGGTTCATCTTTTATG 1800
GluGlnMetSerThrIleGluSerLeuIleGlnLysIleAlaGlyTyrValMetAspAsnArgSerGlyGlySerAspAlaSerGlnAspArgAlaAlaGlyGlyGlySerSerPheMet

1801 AACACTTTAATGGCAGACTCTAAGGGTTCTTCCCAAACGCAACTAGGAAAACTAGCTTTGTTAGCCACAGTGATGACACACTCATCAAAATAAAGGTTCTTCTAACAGAGGGTTTGACGTA 1920
AsnThrLeuMetAlaAspSerLysGlySerSerGlnThrGlnLeuGlyLysLeuAlaLeuAlaThrValMetThrHisSerSerAsnLysGlySerSerAsnArgGlyPheAspVal

1921 GGGACTGTCATGTCATGCTAAGTGGTCTGGCGCGGGAGCCAAAGTATGGGTGCTTCCGGCCTGGCTGGCTTGGCTTCTCAATTCTTTAAGTCAGGTAAACAATCCCAAGGTCAGGGA 2040
GlyThrValMetSerMetLeuSerMetLysGlyGlyGlySerGlnSerMetGlyAlaSerGlyLeuAlaAlaLeuAlaSerGlnPhePheLysSerGlyAsnAsnSerGlnGlnGlyGlnGly

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FIG. 4 CONT.

2041 CAAGTCAAGGTCAAGGTCAAGGTCAAGGTCCTTTACTGCTTTGGCGTCTTTAACTTCATCTTTCATGAATTCCAACAACAATAATCAGCAAGGTCAAAT 2160
GlnGlyGlnGlyGlnGlyGlnGlyGlnGlyGlnGlySerPheThrAlaLeuAlaSerLeuThrSerPheMetAsnSerAsnAsnAsnGlnGlnGlyGlnAsn

22161 CAAAGCTCCGGTGGTTCCCTCTTTGGACCACCTGGCTICTATGGCAAGCTCTTTTATGCATTCCAATAATCAGAACTCCAACAATAGTCAACAGGGCTATAACCAATCTCTATCAAAC 2280
GlnSerSerGlyGlySerSerPheGlyAlaLeuAlaSerMetAlaSerSerPheMetHisSerAsnAsnAsnGlnAsnSerAsnAsnSerGlnGlnGlyTyrAsnGlnSerTyrGlnAsn

2281 GGTAAACAAAATAGTCAAGGTTACAATAATCAACAGTIACCAAGGTGGGTAACCAACAACAACAGGGACAATCTGGTGGTGCTTTTTTCCTCATTTGGCCTCCATGGCTCAAATCT 2400
GlyAsnGlnAsnSerGlnGlyTyrAsnAsnGlnGlnTyrGlnGlnGlyGlyAsnGlyGlyTyrGlnGlnGlnGlnGlnSerGlyGlyAlaPheSerSerLeuAlaSerMetAlaGlnSer

2401 TACTTAGGTGGACAAACTCAATCCAACCAACAGCAATACAAATCAACAAGGCCAAAAACAACAGCAGCAATACCAGCAACAAGGCCAAAACTATCAGCATCAACAACAGGGGTGAGCAG 2520
TyrLeuGlyGlyGlyGlnThrGlnSerAsnGlnGlnGlnTyrAsnGlnGlnGlyGlnAsnAsnGlnGlnGlnTyrGlnGlnGlnGlnGlnAsnTyrGlnHisGlnGlnGlnGlnGln

2521 CAGCAACAAGCCCACTCAGTTCATCTCAGCTTTGGCTTCATGGCAAGTTCCTACCTGGGCAATAAATCCAAATTCAAATTCGAGTTATGGGGCCAGCAACAGGCTAATGAGTATGGTA 2640
GlnGlnGlnGlyHisSerValHisSerGlnLeuTrpLeuProTrpGlnValProThrTrpAlaIleThrProIleGlnIleArgValMetGlyAlaSerAsnArgLeuMetSerMetVal

2661 GACCGCAACAGAAATGGTCAACAGCAATCCAATGAGTACGGGAAGACCCGCAATACGGCGGAAACCCAGAACCTCCATTCAAATTTTCTGSCAACATTTCTCAACAGA 2760
AspArgAsnArgMetValAsnSerAsnProMetSerThrGluAspArgAsnThrAlaGluThrArgThrProMetAspSerThrAsnProSerIlePheLeuAlaThrPheLeuAsnArg

2761 ACAATAACGGCAACCAACGAGTCTGACGATGATTCAGTTGCGCTTCTATCCTTTGTTTACGTAATTTGTTTATATATATAACTTTATTTTTTTTATTAATTTGGGCTGCAAGACAAT 2880
ThrIleThrAlaThrArgThrAlaThrGluArgEnd

2881 TTGTGTGTCAGTGAIGCCCTCAATCTCTTTTGTCTCCATATTTACCAATGTGAGCCCTTTCAAAACAGAGTTGTATCTCTCGCAGGATGCCCTTTTTCGACGTAATGAATGGCATAATTGC 2999

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FIG. 4 CONT.

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3840 GAGAAAGTTGTGGATGCAATCCTCTGTATACCTAAACCCAGCAATATTGTCATATTGAATCCAGCATGCCCTCGTATATGATTACCTTCTCAAAAAACGGTGTCTCTCAAGAAAACTGCG 3959
206 GluLysValValAspAlaPheLeuTyrThrLysProProAsnIleValHisIleGluSerSerMetProSerTyrAsnAspLeuProSerGlnLysThrValSerSerLysLysThrAla
3960 TTAAAAACGAGTGAGAAATGGAGTTACGAATCTCCACTATCTCGATGGTTCITGAGGGGTCTACATACTTTAAGGATTATGGCTTATCAAAAGACCTCTTTAAAGACCCCAACTGGGGCT 4079
246 LeuLysThrSerGluLysTrpSerTyrGluSerProLeuSerArgTrpPheLeuArgGlySerThrTyrPheLysAspTyrGlyLeuSerLysThrSerLeuLysThrProThrGlyAla
4080 CCACAACTGAAGCAAAATGAAATGCTCTCCGGATAAGTAAGGGTTACTTCAATGAGTCAGATATAATGCCTGACGAACGATCGCCCATCTTGGAGTATAATAACACGCCCTCTGGATGCA 4199
286 ProGlnLeuLysGlnMetLysMetLeuSerArgIleSerLysGlyTyrPheAsnGluSerAspIleMetProAspGluArgSerProIleLeuGluTyrAsnAsnThrProLeuAspAla
4200 AATGACAGTGTGAATAACTTGGGTAATACCCAGCCAGATTACAAAATCACATCTTATCGCAACAATAACATCGATCTAATCACGGCAAGACCCCATTCAGTGATATACGGTACTACTGCA 4319
326 AsnAspSerValAsnAsnLeuGlyAsnThrThrProAspSerGlnIleThrSerTyrArgAsnAsnAsnIleAspLeuIleThrAlaArgProHisSerValIleTyrGlyThrThrAla
4320 CAACAAACTTTGGAAACCAACTTCAATGATCATGACTGCAATATAAAGCACTGAGAAACACGAGTTGATAATACCCACCCCATCAAAACCCACTAAAGAAAAAGAAAAAGAGACAA 4439
366 GlnGlnThrLeuGluThrAsnPheAsnAspHisHisAspCysAsnLysSerThrGluLysHisGluLeuIleLeProThrProSerLysProLeuLysLysArgLysLysArgArgGln
4440 AGTAAATGTATCAGCATTACAAACATTTGTACGTTCTAAACCATTTGCCGCTTACTCCAAACTCCAAATATAATGGGGAGGCTAGCGTCCAATTAGGGAAGACATATACAGTTATTACG 4559
406 SerLysMetTyrGlnHisLeuGlnHisLeuSerArgSerLysProLeuProLeuThrProAsnSerLysTyrAsnGlyGluAlaSerValGlnLeuGlyLysThrTyrThrValIleGln
4560 GATTACGAGCCTAGATTACAGACGCAAAATAGAAATCTCGCTGGGIGAAAAAGTTAAATTTCTGGCCACTATACCCATGGATGGTGTCTGGTAGAAAAAGTGTAAATACACAAAGGGTTCT 4679
446 AspTyrGluProArgLeuThrAspGluIleArgIleSerLeuGlyGluLysValLysIleLeuAlaThrHisThrAspGlyTrpCysLeuValGluLysCysAsnThrGlnLysGlySer
4680 ATTACAGTCAGTGTGACGATAAAGATACCTCAATGAACATAGAGGCATTTGTGCCCTGGTACTGCTCCAAAGAAATACGACTGATGAAAAATATATTGACGTTCCGATTAACTCTATACC 4799
486 IleHisValSerValAspAspLysArgTyrLeuAsnGluAspArgGlyIleValProGlyAspCysLeuGlnGluTyrAspEndEnd End End

```

FIGURE 5 10/14

1 GATCTTTTGAATTTTGCATTAAACAGTGAAGAAGGTAGTAGAGACCGTTTCAAAGTCATA 60
-----+-----+-----+-----+-----+
CTAGAAAACCTTAAACGTAATTTGTCACTTCTTCCATCATCTCTGGCAAAGTTTCAGTAT
10 30 50

61 ACAGAGTTTTTAGGTAGAGGTGCCATCAGTTATTCTGACATCACTATTTAATGATGGATC 120
-----+-----+-----+-----+-----+
TGTCTCAAAAATCCATCTCCACGGTAGTCAATAAGACTGTAGTGATAAATTACTACCTAG
70 90 110

121 ATAACGATCTATTGTGCCCGCCGCGTCACAAATGCGCCCCGAAGTTGTCGCGAAGTTAAT 180
-----+-----+-----+-----+-----+
TATTGCTAGATAACACGGGCGGCGCAGTGTTCACGCGGGGCTTGAACAGCGCTTCAATTA
130 150 170

181 CTGAAACATATATGTTACCTACTGAAACAGCGCATGTTGGAAAAGACAAAGGTGAAGACG 240
-----+-----+-----+-----+-----+
GACTTTGTATATACAATGGATGACTTTGTGCGGTACAACCTTTTCTGTTTCCACTTCTGC
190 210 230

241 AAGTTGTATATTTAAGATAGACCCTTTATACATCCTTTTGAAAAAATTATTAATGTGGCA 300
-----+-----+-----+-----+-----+
TTCAACATATAAATTCTATCTGGGAAATATGTAGGAAAACCTTTTTAATAATTACACCGT
250 270 290

301 ACCGTCCTTTTATTTGACAAAGTATCTTTTTTCTTTGTGAAACCAATTTTAGGTTTTCTTG 360
-----+-----+-----+-----+-----+
TGGCAGAAAATAAACTGTTTCATAGAAAAAGAAACACTTTGGTTAAAATCCAAAAGAAC
310 330 350

MetPheLysThrSerTyr

361 TTATAGTAAAGTTCTTAAGAAAAAGACAAGAAAACCCCTTGCGATGTTTAAGACTTCATAT 420
-----+-----+-----+-----+-----+
AATATCATTCAAGAATTCTTTTTCTGTTCTTTTGGGGAACGCTACAAATTCTGAAGTATA
370 390 410

AsnLeuTyrAspLeuAsnTyrProLysAsnAspSerLeuThrProIleArgAspTyrLys

421 AACTTGATACGATTGAACTATCCGAAAAATGATTCATTAACGCCAATAAGAGACTACAAA 480
-----+-----+-----+-----+-----+
TTGAACATGCTAACTTGATAGGCTTTTTACTAAGTAATTGCGGTATTCTCTGATGTTT
430 450 470

AsnAspTyrPheHisLysAsnAspAspLysLeuProGluIleValArgLysProThrArg

481 AATGACTATTTTCATAAAAATGATGACAAATTACCAGAAATTGTTAGAAAACCTACGAGA 540
-----+-----+-----+-----+-----+
TTACTGATAAAAGTATTTTTACTACTGTTTAATGGTCTTTAACAATCTTTTGGATGCTCT
490 510 530

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FIGURE 5 (CONT'D)

LysLeuSerLysHisGluAsnLysLeuAsnAspLysLysPheThrAsnLysArgProAla
 541 AAGTTATCGAAACATGAAAACAAACTCAACGATAAAAAATTCACGAATAAACGACCAGCA 600
 -----+-----+-----+-----+-----+-----+-----+
 TTCAATAGCTTTGTACTTTTGTGTTGAGTTGCTATTTTTTAAGTGCTTATTTGCTGGTCGT
 550 570 590

SerLeuAspLeuHisSerIleValGluSerLeuSerAsnLysLysIleTyrSerProIle
 601 AGTCTGGACTTGCATTCTATAGTGGAGAGCCTGAGCAATAAAAAATTTACTCTCCTATT 660
 -----+-----+-----+-----+-----+-----+-----+
 TCAGACCTGAACGTAAGATATCACCTCTCGGACTCGTTATTTTTTAAATGAGAGGATAA
 610 630 650

AsnThrGluIlePheGlnAsnValValArgLeuAsnLeuSerProGlnIleProAsnSer
 661 AACACAGAGATATTTCAAATGTCGTGAGACTGAATTTGAGCCCTCAGATTCCTCAATTCT 720
 -----+-----+-----+-----+-----+-----+-----+
 TTGTGTCTCTATAAAGTTTTACAGCACTCTGACTTAACTCGGGAGTCTAAGGGTTAAGA
 670 690 710

ProHisGluGlyCysLysPheTyrLysIleValGlnGluPheTyrLeuSerGluValGlu
 721 CCTCACGAGGGATGCAATTTTATAAAATCGTACAGGAGTTTTACCTCTCTGAAGTGGAA 780
 -----+-----+-----+-----+-----+-----+-----+
 GGAGTGCTCCCTACGTTTAAATATTTTAGCATGTCTCAAATGGAGAGACTTCACCTT
 730 750 770

TyrTyrAsnAsnLeuLeuThrAlaAsnAsnValTyrArgLysAlaLeuAsnSerAspPro
 781 TATTACAATAATTGTTAACC^{HaeI}CGCAAATAACGTATACAGAAAGGCATTGAATAGTGATCCA 840
 -----+-----+-----+-----+-----+-----+-----+
 ATAATGTTATTAAACAATTGGCGTTTATTGCATATGTCTTCCGTAACCTTATCACTAGGT
 790 810 830

ArgPheLysAsnLysLeuValLysLeuAspSerSerAspGluLeuLeuLeuPheGlyAsn
 841 AGATTCAAGAATAAACTTGTCAAGCTTGATTCAAGTGACGAGCTATTGCTTTTTTGGGAAC 900
 -----+-----+-----+-----+-----+-----+-----+
 TCTAAGTTCTTATTTGAACAGTTCGAACTAAGTTCAGTCTCGATAACGAAAAACCTTG
 850 870 890

IleAspThrIleAlaSerIleSerLysIleLeuValThrAlaIleLysAspLeuArgLeu
 901 ATTGACACTATTGCGTCAATCAGCAAAATACTGGTAACGGCAATAAAAGACCTACGGTTA 960
 -----+-----+-----+-----+-----+-----+-----+
 TAACTGTGATAACGCAGTTAGTCGTTTTATGACCATTGCCGTTATTTTCTGGATGCCAAT
 910 930 950

AlaLysGlnArgGlyLysMetLeuAspAlaAsnGluTrpGlnLysIleLeuThrLysAsn
 961 GCCAAGCAACGTGGGAAAATGTTGGATGCGAATGAATGGCAAAAGATATTGACCAAAAAT 1020
 -----+-----+-----+-----+-----+-----+-----+
 CGGTTTCGTTGCACCCTTTTACAACCTACGCTTACTTACCGTTTTCTATACTGGTTTTTA
 970 990 1010

FIGURE 5 (CONT'D)

GluValGlnGlnGlnLeuTyrSerThrPheAspIleSerGluAlaPheGluGlnHisLeu 12/14
 1021 GAGGTACAACAGCAGCTATATTCAACTTTTGTATTTTCAGAGGCGTTCGAGCAACATTTG 1080
 -----+-----+-----+-----+-----+-----+-----+
 CTCCATGTTGTCGTCGATATAAGTTGAAACTATAAAGTCTCCGCAAGCTCGTTGTAAAC
 1030 1050 1070
 LeuArgIleLysSerThrTyrThrSerTyrPheValSerHisGlnLysGlnMetGluLeu
 1081 TTAAGAATCAAATCCACCTACACAAGCTATTTTGTAGCCACCAAAAACAAATGGAACATA 1140
 -----+-----+-----+-----+-----+-----+-----+
 AATTCTTAGTTTAGGTGGATGTGTTTCGATAAAACAATCGGTGGTTTTTGTTTACCTTGAT
 1090 1110 1130
 PheThrThrLeuArgMetAsnLysAsnHisPhePheAsnLysTrpTyrGluTyrCysLeu
 1141 TTTACTACATTAAGGATGAATAAGAATCATTTTTTTAACAAGTGGTATGAATATTGTTTA 1200
 -----+-----+-----+-----+-----+-----+-----+
 AAATGATGTAATTCCTACTTATTCTTAGTAAAAAAATTGTTCCACATACTTATAACAAAT
 1150 1170 1190
 LysGluSerGlyCysIleLysLeuGluAspIleLeuLysSerProMetLysArgLeuThr
 1201 AAAGAGAGTGGATGTATAAAGTTAGAGGACATATTGAAAAGCCCGATGAAAAGACTGACT 1260
 -----+-----+-----+-----+-----+-----+-----+
 TTTCTCTCACCTACATATTTCAATCTCCTGTATAACTTTTCGGGCTACTTTTCTGACTGA
 1210 1230 1250
 GlnTrpIleAspThrLeuGluThrLeuGluSerCysTyrGluAspIleLeuSerProGlu
 1261 CAGTGGATTGATACTTTGGAACTTTGGAAAGCTGTTACGAAGATATTCTTTCCGCCAGAA 1320
 -----+-----+-----+-----+-----+-----+-----+
 GTCACCTAACTATGAAACCTTTGAAACCTTTTCGACAATGCTTCTATAAGAAAGCGGTCTT
 1270 1290 1310
 LeuGlyLeuLysLeuSerProThrArgArgLysTyrSerLeuPheSerAsnLysLeuGlu
 1321 TTGGGCTTGAAACTAAGCCCGACAAGAAGAAAATATTCTTTATTTTCCAATAAGTTAGAA 1380
 -----+-----+-----+-----+-----+-----+-----+
 AACCCGAACCTTGATTTCGGGCTGTTCTTTCTTTTATAAGAAATAAAAGGTTATTCAATCTT
 1330 1350 1370
 ThrGluValSerGluTyrLysSerAsnSerMetTyrAsnPheSerLeuThrProSerGlu
 1381 ACCGAGGTCTCCGAGTATAAGAGTAATTCATGTATAATTTTCAGTTTAACCCCATCAGAG 1440
 -----+-----+-----+-----+-----+-----+-----+
 TGGCTCCAGAGGCTCATATTCTCATTAAGGTACATATTAAAGTCAAATTGGGGTAGTCTC
 1390 1410 1430
 IleIleGlnSerTyrAspGluAspGlnPheThrHisLeuLeuLysProProAspLysGln
 1441 ATTATACAAAGTTATGATGAAGATCAGTTTACACACCTTTTAAAACCCCCAGACAAACAA 1500
 -----+-----+-----+-----+-----+-----+-----+
 TAATATGTTTCAATCTACTTCTAGTCAAAATGTGTGGAAAATTTTGGGGGTCTGTTTGT
 1450 1470 1490
 AsnLysAsnIleCysAsnAlaSerArgGlnGluSerAsnLeuAspAsnSerArgValPro

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FIGURE 5 (CONT'D)

1501	AATAAAAATATATGTAATGCATCTCGACAAGAGAGTAATTTGGATAATAGTAGAGTTCCT -----+-----+-----+-----+-----+-----+-----+ TTATTTTTTATATACATTACGTAGAGCTGTTCTCTCATTAAACCTATTATCATCTCAAGGA 1510 1530 1550	13/14 1560
1561	SerLeuLeuSerGlySerSerSerTyrTyrSerAspValSerGlyLeuGluIleValThr TCTCTTCTTTCTGGATCATCGAGTTACTACTCAGATGTATCAGGGCTAGAAATTGTCACT -----+-----+-----+-----+-----+-----+-----+ AGAGAAGAAAGACCTAGTAGCTCAATGATGAGTCTACATAGTCCCGATCTTTAACAGTGA 1570 1590 1610	1620
1621	AsnThrSerThrAlaSerAlaGluMetIleAsnLeuLysMetAspGluGluThrGluPhe AATACTTCAACTGCCCTCAGCTGAGATGATAAATCTAAAAATGGATGAAGAAACAGAATTT -----+-----+-----+-----+-----+-----+-----+ TTATGAAGTTGACGGAGTCGACTCTACTATTTAGATTTTACCTACTTCTTTGTCTTAAA 1630 1650 1670	1680
1681	PheThrLeuAlaAspHisIleSerLysPheLysLysValMetLysGlyLeuLeuGluLeu TTTACATTGGCAGATCACATCAGTAAATTCAAGAAAGTAATGAAAGGTTTGTTAGAATTA -----+-----+-----+-----+-----+-----+-----+ AAATGTAACCGTCTAGTGTAGTCATTTAAGTTCTTTCATTACTTTCCAAACAATCTTAAT 1690 1710 1730	1740
1741	LysLysAsnLeuLeuLysAsnAspLeuSerGlyIleIleAspIleSerLeuArgArgIle AAAAAAGAAATTTATTGAAAAACGATCTGTCAGGCATTATTGATATCAGTTTAAGAAGAATA -----+-----+-----+-----+-----+-----+-----+ TTTTCTTAAATAACTTTTTGCTAGACAGTCCGTAATAACTATAGTCAAATTCTTCTTAT 1750 1770 1790	1800
1801	AsnAlaTrpLysLysValIleGluCysGluArgProSerGlyAlaPhePheAlaHisAsp AATGCATGGAAAAAGGTGATCGAGTGCGAACGCCCTTCTGGTGCATTTTTTGCGCACGAT -----+-----+-----+-----+-----+-----+-----+ TTACGTACCTTTTTCCACTAGCTCACGCTTGCGGAAGACCACGTAAAAAACGCGTGCTA 1810 1830 1850	1860
1861	AsnLeuIleSerThrMetCysSerSerTyrIleAspLysLeuHisGluGlnLysAsnGln AACTTAATATCGACCATGTGTTCTTCGTACATAGATAAACTGCATGAACAAAAAATCAA -----+-----+-----+-----+-----+-----+-----+ TTGAATTATAGCTGGTACACAAGAAGCATGTATCTATTTGACGTACTTGTTTTTTTAGTT 1870 1890 1910	1920
1921	ValThrIleLeuLysLeuThrGluLeuGluThrAspValMetAsnProLeuGluArgIle GTAACAATTTTGAAACTCACAGAGCTCGAAACAGATGTGATGAACCCACTTGAAAGAATC -----+-----+-----+-----+-----+-----+-----+ CATTGTAAAACTTTGAGTGTCTCGAGCTTTGTCTACACTACTTGGGTGAACCTTCTTAG 1930 1950 1970	1980
1981	IleAlaHisCysThrThrValLysSerLysLeuLysAspLeuGlnAlaTyrMetLeuPhe ATAGCCCATTTGTAATACTACCGTTAAAAGCAAACCTAAAAGATTTGCAAGCTTACATGTTATTT -----+-----+-----+-----+-----+-----+-----+	2040

FIGURE 5 (CONT'D)

14/14

TATCGGGTAACATGATGGCAATTTTCGTTTGATTTTCTAAACGTTTGAATGTACAATAAA
 1990 2010 2030

LeuGlnGluLysLysAlaAsnValArgAspIleLysArgAspLeuLeuGlyMetHisPhe
 TTACAAGAAAAAAGCAAATGTGCGAGATATTAAACGTGACTTGTTGGGAATGCATTTT
 2041 2100
 AATGTTCTTTTTTTCGTTTACACGCTCTATAATTTGCACTGAACAACCCTTACGTAAAG
 2050 2070 2090

GlnAsnLeuGlnAsnGlnMetLysArgGluLeuProValPheIleThrLeuIleProArg
 CAAAACCTGCAAAACCAGATGAAAAGGGAATTACCGGTCTTTATTACTTTGATCCACGA
 2101 2160
 GTTTTGGACGTTTTGGTCTACTTTCCCTTAATGGCCAGAAATAATGAACTAGGGTGCT
 2110 2130 2150

TyrTyrArgMetTyrLeuValGluLeuTyrGlnSerLeuLeuLysIlePheGlyAsnHis
 TACTATCGAATGTATCTTGTTGAACTATATCAAAGTCTTCTTAAATATTTGGAAATCAT
 2161 2220
 ATGATAGCTTACATAGAACAACCTTGATATAGTTTCAGAAGAATTTTATAAACCTTTAGTA
 2170 2190 2210

CysTrpTrpLysLysIleProAlaLysArgSer
 TGCTGGTGAAAAAATACCTGCAAAAAGATCTTGAAATATGTCTCTTAATGACTCTAT
 2221 2280
 ACACACCTTTTTTATGGACGTTTTTCTAGAATTTTATACAGAGAATTACTGAGATA
 2230 2250 2270

AGCTACCGGCCAAATTAAAAATCTTGATATTTTGCAGTGTTATTCTAAATCAGATATAT
 2281 2340
 TCGATGGCCGGTTTAATTTTGAAGCTATAAACGTCACAATAAGATTTAGTGCTATATA
 2290 2310 2330

ATGACAAAACGCATGGTAAGAAAAGATTGGCCTTTCCCTGGAGACCCTAGTGGAAGCCGT
 2341 2400
 TACTGTTTTGCGTACCATTCTTTTCTAACCGGAAAGGGACCTCTGGGATCACCTTCGGCA
 2350 2370 2390

GTTGTGAGAAAACCTTTTCGAACCTTAAACAAAAGAGTATATTTAGCTTATAGTTTTAGAA
 2401 2460
 CAACAGTCTTTTGAAAAGCTTGAAATTGTTTTCTCATATAAATCGAATATCAAAAATCTT
 2410 2430 2450

TGTTTTGTTTTGTTTTTACTAAAGTAGTACT
 2461 2492
 ACAAACAAAACAAAATGATTTTCATCATGA
 2470 2490

INTERNATIONAL SEARCH REPORT

International Application No PCT/US88/02129

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁴ : C 12 N 15/00, C 12 N 1/16, C 12 P 21/02																							
II. FIELDS SEARCHED <div style="text-align: center;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border: none;"> <tr> <td style="width: 30%; border: none;">Classification System</td> <td style="border: none;">Classification Symbols</td> </tr> <tr> <td style="border: none; text-align: center; padding: 10px;">IPC⁴</td> <td style="border: none; text-align: center; padding: 10px;">C 12 N; C 12 P</td> </tr> </table> <div style="text-align: center; padding: 5px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *</div>			Classification System	Classification Symbols	IPC ⁴	C 12 N; C 12 P																	
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III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁸ <table style="width: 100%; border: none;"> <tr> <th style="width: 10%; border: none;">Category *</th> <th style="width: 60%; border: none;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 30%; border: none;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="border: none; text-align: center; vertical-align: top;">Y</td> <td style="border: none; vertical-align: top;">Molecular and Cellular Biology, volume 7, No. 2, February 1987, p 749-759, (SCOTT W. VAN ARSDELL et al), "The Yeast Repeated Element Sigma Contains a Hormone-Inducible Promoter".</td> <td style="border: none; vertical-align: top;">1-4, 13-14, 23-26, 35, 36</td> </tr> <tr> <td style="border: none; text-align: center; vertical-align: top;">P, Y</td> <td style="border: none; vertical-align: top;">Chemical Abstracts, Vol 107 (1987) abstract No 212743x, (Trueheart), Mol.Cell.Biol.1987, 7(7), 2316-28 (Eng). *Abstract*</td> <td style="border: none; vertical-align: top;">1-41</td> </tr> <tr> <td style="border: none; text-align: center; vertical-align: top;">P, Y</td> <td style="border: none; vertical-align: top;">Chemical Abstracts, Vol 107 (1987) abstract No 128287u, (McCaffrey) Mol.Cell.Biol. 1987, 7(8), 2680-90 (Eng). *Abstract*</td> <td style="border: none; vertical-align: top;">1-41</td> </tr> <tr> <td style="border: none; text-align: center; vertical-align: top;">P, Y</td> <td style="border: none; vertical-align: top;">Chemical Abstracts, Vol 108 (1988) abstract No 217221f, (Nonato, Roberto V.) Biochem.Biophys.Res.Comm. 1988, 152(1), 76-82 (Eng). *Abstract*</td> <td style="border: none; vertical-align: top;">1-4, 13-14, 23-26, 35, 36</td> </tr> <tr> <td style="border: none; text-align: center; vertical-align: top;">Y</td> <td style="border: none; vertical-align: top;">EP, A2, 0 183 350 (IMMUNEX CORPORATION) 4 June 1986 *Claim 7*</td> <td style="border: none; vertical-align: top;">1-4, 13-14, 23-26, 35, 36</td> </tr> <tr> <td colspan="3" style="border: none; text-align: right; padding-right: 20px;">.../...</td> </tr> </table> <div style="font-size: small; padding: 5px;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div>			Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	Y	Molecular and Cellular Biology, volume 7, No. 2, February 1987, p 749-759, (SCOTT W. VAN ARSDELL et al), "The Yeast Repeated Element Sigma Contains a Hormone-Inducible Promoter".	1-4, 13-14, 23-26, 35, 36	P, Y	Chemical Abstracts, Vol 107 (1987) abstract No 212743x, (Trueheart), Mol.Cell.Biol.1987, 7(7), 2316-28 (Eng). *Abstract*	1-41	P, Y	Chemical Abstracts, Vol 107 (1987) abstract No 128287u, (McCaffrey) Mol.Cell.Biol. 1987, 7(8), 2680-90 (Eng). *Abstract*	1-41	P, Y	Chemical Abstracts, Vol 108 (1988) abstract No 217221f, (Nonato, Roberto V.) Biochem.Biophys.Res.Comm. 1988, 152(1), 76-82 (Eng). *Abstract*	1-4, 13-14, 23-26, 35, 36	Y	EP, A2, 0 183 350 (IMMUNEX CORPORATION) 4 June 1986 *Claim 7*	1-4, 13-14, 23-26, 35, 36	.../...		
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Y	EP, A2, 0 183 350 (IMMUNEX CORPORATION) 4 June 1986 *Claim 7*	1-4, 13-14, 23-26, 35, 36																					
.../...																							
IV. CERTIFICATION <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none; vertical-align: top;"> Date of the Actual Completion of the International Search 23th September 1988 </td> <td style="width: 50%; border: none; vertical-align: top;"> Date of Mailing of this International Search Report 11 NOV 1988 </td> </tr> <tr> <td style="border: none; vertical-align: top;"> International Searching Authority EUROPEAN PATENT OFFICE </td> <td style="border: none; vertical-align: top;"> Signature of Authorized Officer P.C.G. VAN-DER PUTTEN </td> </tr> </table>			Date of the Actual Completion of the International Search 23th September 1988	Date of Mailing of this International Search Report 11 NOV 1988	International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer P.C.G. VAN-DER PUTTEN																	
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Chemical Abstracts, Vol 103 (1985) abstract No 208060x, (Miyajima Atusushi) Gene 1985, 37(1-3), 155-61 (Eng). *Abstract*	1-41
A	EP, A2, 0 123 544 (GENENTECH. INC.) 31 October 1984 *The whole document*	1-41
A	Chemical Abstracts, Vol 104 (1986) abstract No 220092n, (Vlasuk George P) J. Biol.Chem. 1986, 261(11), 4789-96 (Eng). *Abstract*	1-41
A	Chemical Abstracts, Vol 104 (1986) abstract No 16037u, (Singh Arjun et al) Genet:New Front.,Proc.Int.Congr., 15th 1983 (Pub.1984). 2, 33-9 (Eng). *Abstract*	1-41
A	US, A, 4 615 974 (KINGSMAN et al) 7 October 1986 *The whole document*	1-41

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

PCT/US88/02129

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This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 01/09/88
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EP-A- 0123544	31-10-84	JP-A- 60041487	05-03-85
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